An alternative approach based on microfluidics to study drug metabolism and toxicity using liver and intestinal tissue

Midwoud, Paul Marcel van

8

Biocompatibility and Adsorption Properties of Plastics to Fabricate Microfluidic Devices for Cell Experiments

Paul M. van Midwoud
Arnout H.J. Janse
Marjolijn T. Merema
Geny M.M. Groothuis
Elisabeth Verpoorte

Submitted

Abstract

The elastomer polydimethylsiloxane (PDMS) is often used as polymer to fabricate microfluidic devices for cell culture experiments. The ease to fabricate and the low cost are ideal for rapid prototyping. However, PDMS has the disadvantage that the surface is hydrophobic and small compounds can penetrate into PDMS. Therefore PDMS can adsorb hydrophobic compounds and absorb small hydrophobic compounds, whereas surface treatment to reduce the hydrophobicity is not successful. Instead of PDMS, thermoplastic polymers (plastics) can be used to produce microfluidic devices in high quantities in the industry at low costs. They allow easy surface treatment, and most of them are transparent and biocompatible. This study focuses on the possibilities to fabricate biocompatible microfluidic devices with low absorptive characteristics which can be used to culture cells or tissue. The surface of the plastics polymethylmethacrylate (PMMA), polystyrene (PS), polycarbonate (PC), and cyclic olefin copolymer (COC, Zeonor®) was treated with oxygen plasma or ultraviolet light (UV) and ozone to oxidize the surface, thereby making it more hydrophilic. By contact angle measurements it was shown that 15-30 minutes of treatment with UV ozone or 30-60 seconds with oxygen plasma resulted in a surface with hydrophilic character. The hydrophilic surface was more stable after UV ozone (more than one week for all plastics tested) than after oxygen plasma treatment. The effect of surface treatment on adsorption of compounds was tested by flushing 7-ethoxycoumarin and testosterone with its metabolites through microfluidic devices, made of these 4 plastics with a hot embosser. After treatment with UV ozone, no adsorption of the hydrophobic substrates or their metabolites occurred on PC, PS and COC. However, a substantial amount of the hydrophobic compounds adhered to PMMA, as well as to PDMS. The effect of UV ozone treatment on the biocompatibility of the plastics was assessed by culturing human hepatoma HepG2 cells on the UV ozone treated surfaces. PDMS, PC, PS and COC were biocompatible after treatment, however, culturing on PMMA resulted in loss of viability. In conclusion, the plastics PC, PS and COC can be used to develop biocompatible microfluidic devices with a low adsorption profile after UV ozone treatment for cell culture experiments. However, when liquid chromatography is to be incorporated on the device, PS is less suitable due to the low solvent resistance compared to PC and COC. Production of UV ozone treated microfluidic devices from PC or COC will result in biocompatible low-cost devices with good surface characteristics which can easily be produced in large batches in the industry.

Introduction

Microfluidic technology has been utilized to develop advanced cell culture models which are useful *in vitro* tools to better understand human biology and disease.[1, 2] Various *in vitro* models have been developed incorporating endothelial, liver, lung, fat, bone tissue (cells) and other types of cells or tissue.[3] By applying microfluidic technology for cell culturing, the complex architecture of organs can be mimicked,[4] and medium flow can be applied and altered during an experiment mimicking the natural cell environment.[1, 2] Mostly, those new *in vitro* chips have been developed in polydimethylsiloxane (PDMS). PDMS has the advantages of biocompatibility, low cost, optical transparency, ease of fabrication, and gas (oxygen and carbon dioxide) permeability.[5] For these reasons PDMS is ideal as polymer to develop prototypes for the incorporation of cells or tissue on microfluidic chips. Recently, we developed a microfluidic-based PDMS device for the incubation of precision-cut liver slices.[6] The tissue slices were viable and metabolically active in the microdevice, however, adsorption of the hydrophobic substrates 7-ethoxycoumarin[6] and testosterone and its metabolites (Chapter 6) was observed, which prevented quantitative assessment of drug metabolizing capacity.

PDMS has a hydrophobic character, which is a disadvantage when studying drug metabolism and toxicity, since hydrophobic substrates and metabolites can interact with the material. Due to the high surface-to-volume ratio in microfluidic devices, the interaction between compounds present in the microchannels and the channel wall material will be more pronounced compared to lower surface-to-volume ratios (conventional systems). Therefore adsorption of hydrophobic compounds from the liquid to the PDMS, is relatively high.[7, 8] This effect is even used in the field of solid phase extraction in so called stir-bar sorptive extraction (SBSE).[9, 10] Stir-bar rods coated with PDMS are used to pre-concentrate and extract organic compounds from aqueous solutions, like sea water for example. PDMS has a porous structure which will also result in absorption of small hydrophobic compounds into PDMS.[8] Coatings with a hydrophilic compound can be applied to overcome these problems,[5] however, stability of coatings is problematic and coatings might influence cell responses.[11]

Various materials can be used to produce microfluidic chips like glass, silicon, plastics, and flexible polymers. However, glass and silicon are rather expensive compared to the other materials, and flexible polymers like PDMS have the disadvantage of adsorbing/absorbing hydrophobic compounds as mentioned above. Therefore plastics might be a good alternative to PDMS for the development of microfluidic chips. In conventional cell culture experiments, plastics like polycarbonate and polystyrene are also used as material to produce culture flasks and well plates. These plastics are hydrophobic in their native form, however, it is easy to reduce their hydrophobicity by oxidizing the surface.[12] In conventional systems, the flasks are so-called tissue culture treated, which means that the surfaces are oxidized.[13]

In general, in every laboratory that fabricates chips an UV ozone or oxygen plasma apparatus is usually available, which can be used to oxidize substrates resulting in hydrophilic surfaces. However, after both UV ozone or oxygen plasma treatment, the substrate will recover from its oxidative situation and recover its hydrophobic state again, so called hydrophobic recovery.[14] This hydrophobic recovery is known for PDMS,[15] however, less information about the hydrophobic recovery of plastics is available in the literature.

By making the surface hydrophilic, hydrophobic compounds will not adsorb onto the material, which makes them suitable for metabolism and toxicity studies with hydrophobic compounds. However, extremely hydrophilic surfaces are charged, which may adsorb charged compounds. Therefore a balance is needed in surface hydrophobicity. Another issue of concern is that surface treatment by UV ozone might be toxic to cells or tissue, due to products, like peroxides, formed on the surface during or after treatment.

The aim of this study is to test several plastics for their applicability as alternative material to PDMS for the incubation and cultivation of cells and tissue without significant adsorption of hydrophobic compounds present in the medium or formed by the cells (e.g. metabolites). A proper plastic should: 1) be suitable to produce multiple chips in a high-throughput manner, 2) not adsorb hydrophobic compounds, and 3) not be toxic to cells after surface treatment to reduce hydrophobicity.

In this study, polycarbonate (PC), polystyrene (PS), polymethylmethacrylate (PMMA), and cyclic olefin copolymer (COC, Zeonor®) are tested. All polymers are thermoplastics and therefore can be used in injection molding or hot embosser to produce chips in bulk in the industry.[16, 17] The plastic surfaces are altered using UV ozone and oxygen plasma treatment, and the hydrophilicity was assessed by measuring the contact angle of water on the surface (angle of liquid-to-solid surface).[18] Hydrophobic recoveries were also measured one and four weeks after treatment. The adsorption of 7-ethoxycoumarin (7-EC) and testosterone (TT) and their liver specific metabolites were measured after treatment. 7-EC and TT are often used in cell culture experiments as model drugs to assess phase I metabolism, and testosterone and its metabolite androstenedione are relatively hydrophobic compounds which adsorb onto PDMS. Finally, as UV ozone appeared somewhat superior to plasma oxygen treatment, the toxicity of the plastics after UV ozone treatment was assessed by the cultivation of liver carcinoma cell line (HepG2) on the substrates for 24 hours. The toxicity was measured by incubating cells after 24 hours with propidium iodide and acridine orange, which stains the dead cells red and the viable cells green, respectively.

In this report it is described how plastic surfaces can be treated to prevent adsorption of hydrophobic compounds, and which plastic is suitable as replacement for PDMS as material to fabricate microfluidic chips for cell or tissue culture.

Materials and Methods

Chemicals and materials

Polycarbonate and polymethylmethacrylate, both 2 mm thick, were obtained from ERIKS Kunststoffen (Leek, The Netherlands). Polystyrene (2 mm thick) was purchased from Kunststofshop (Zevenaar, The Netherlands), and cyclic olefin copolymer (1-2 mm thick, Zeonor®) was obtained from microfluidic chipshop (Jena, Germany). All polymers were used as received and sawn to desired dimensions in-house. 7-Ethoxycoumarin, testosterone, propidium iodide, acridine orange and penicillin/streptomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum was obtained from Gibco (Paisley, UK). Polydimethylsiloxane was obtained as Sylgard 184 pre-polymer and curing agent from Dow Corning (Mavom BV, The Netherlands).

Surface treatment

The hydrophobicity of native plastic surfaces was altered by oxidizing the surface using UV ozone or oxygen plasma. The UV ozone exposure of substrates was performed using an ultraviolet ozone cleaning system (UVOCS, Lansdale, PA, USA). The apparatus contained a low-pressure mercury UV-light, generating UV-emissions at 185 and 254 nm wavelengths. Samples of PMMA, PC, PS, COC, and PDMS with dimensions of 15 mm x 30 mm (L x W) were exposed to UV ozone for 15, 30, 45, and 60 minutes. In addition, samples of PMMA, PC, PS, COC, and PDMS were treated with oxygen plasma using a PDC-002 Harrick Scientific's Plasma Cleaner (Ithaca, NY, USA) for 30, 60, 90 and 120 seconds. The effect of exposure on hydrophobicity was assessed by measuring the contact angle of water with the surface.

Contact angle measurements

When surface is hydrophilic, a water droplet would occupy as large surface area of the material as possible, resulting in a low contact angle. On a hydrophobic surface a water droplet appears, makes little contact with the surface and has a high contact angle. Contact angles were measured using an OCA 20 video-based contact angle meter from Dataphysics (Filderstadt, Germany). Per experiment, three pieces of the same substrate were used on which three times the contact angle was measured (nine measurements per plastic per treatment method). Native substrates were measured and substrates after UV ozone and oxygen plasma treatment. In both cases contact angles of substrates were also measured one and four weeks after treatment. Nine samples per plastic substrate were oxidized, three pieces were used directly after treatment and the rest was aged in air. These substrates were used to measure the hydrophobic recovery of the plastics. It is known that PDMS shows hydrophobic recovery.[19] However, it is unknown to what extend the plastics exhibit this hydrophobic recovery effect. This effect has been studied for a few plastics after oxygen

plasma treatment,[20, 21] however, to the best of our knowledge the hydrophobic recovery has not been studied for the plastics tested in this study after UV ozone treatment.

PDMS and plastic substrates were only used once for contact angle measurements and disposed after the analysis.

Adsorption of hydrophobic compounds

The adsorption of compounds onto the plastic substrates was measured by flushing medium containing substrate and metabolites through the chip. For this purpose chips were produced using a hot embosser (Dr. Collin GmbH, Ebersberg, Germany). A CNC-milled brass mold with the structure as depicted in Figure 1 was produced in-house (accuracy of < 1 µm). The structure was 200 µm thick, and had a length of 10 mm in total. The channels were 500 µm wide and the chamber had a radius of 2 mm. The total volume of the device was approximately 3.5 µL. The chips were cleaned with isopropanol and ultrapure water before the hot-embossing procedure. The structures were imprinted in the plastics using the parameters given in Table 1. The program started at an initial temperature of 25°C and raised to the hot embossing temperature with a ramp of 5°C/min for all plastics. Subsequently, pressure (see Table 1) is applied for 10 minutes. Thereafter, the temperature is cooled down to 60°C with a ramp of 12°C/min, and de-embossing occurred at 60°C. Openings of Ø1.6 mm at the in- and outlet were drilled for the insertion of tubing. Thereafter each channel was irreversibly sealed using a cover chip of the same material by thermal bonding using the hot embosser. The temperature and pressure used to bond two pieces of plastics are also given in Table 1. Here the temperature was also elevated with 5°C/min to the bonding temperature, and the pressure was applied for 10 minutes. Afterwards, the substrates were cooled down to 25°C with a ramp of 12°C/min.

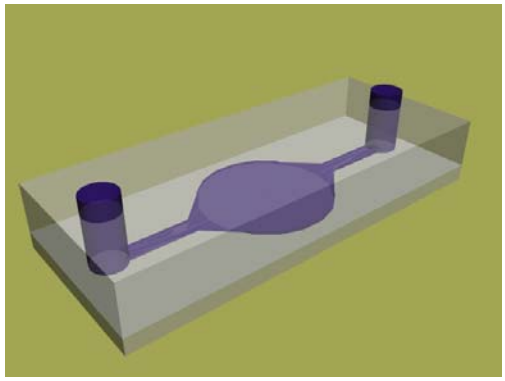

Figure 1. Schematic view of the chip used for adsorption and cell culture studies. The chamber dimension is Ø4 mm x 200 µm (total volume ~3.5 µL) and the flow rate of medium is 5 µL/min.

Table 1. Hot embossing parameters for structure patterning and thermal bonding of plastic substrates.

Plastic	*Glass transition temperature*	*Hot-embossing*		*Bonding*	
		Temperature	Pressure	Temperature	Pressure
PC	145°C[22]	150°C	30 bar	135°C	15 bar
PS	103°C[22]	110°C	20 bar	90°C	15 bar
PMMA	117°C[22]	130°C	10 bar	90°C	10 bar
COC	105°C[23]	110°C	10 bar	95°C	10 bar

Only the production of PDMS devices was different compared to plastic devices. Devices were produced by mixing prepolymer and curing agent in a 10:1 mass ratio, and pouring it over the mold with Ø1.5 mm stainless steel rods placed at the in- and outlet positions for the insertion of tubing during perfusion. The PDMS was left at room temperature for 30 minutes to remove air bubbles which were trapped in PDMS. Thereafter, the temperature was increased to 45°C using a hot plate to cure the PDMS overnight. After curing, the chip was removed from the mold and was bonded to another flat piece of PDMS, which was pretreated in the UV ozone for 60 minutes. An irreversible bond was obtained by placing both substrates in the UVOCS for 15 minutes, and after treatment brought them immediately into contact and left to covalently react with each other, as described before.[6]

Peek tubing (Da Vinci BV, Rotterdam, The Netherlands) were inserted in the chips and connected to syringes filled with substrate and metabolites. 7-Ethoxycoumarin (7-EC) and testosterone (TT) with its metabolites were used as compounds. These solutions were obtained by incubating precision-cut liver slices in well plates with 500 µM 7-EC or 250 µM TT in William's medium E for three hours, as described before.[6] After three hours of incubation, the medium was collected and centrifuged to remove cell debris. Syringes were filled with this medium pre-equilibrated with carbogen gas (95% O_2 / 5% CO_2) at 37°C which contained the substrate with liver specific metabolites. The syringes and the chips were placed in an incubator set at 37°C, and the chips were flushed for two hours with the medium at a flow rate of 5 µL/min. The adsorption experiments were performed in triplicate on three different chips made from the same material. Fractions collected at the outlet were stored at -20°C until analysis. The 7-EC was analyzed using an HPLC with UV-detector as described before.[6] TT with its metabolites were first preconcentrated using liquid-liquid extraction with dichloroform, and the extracted fraction was analyzed using an HPLC with UV detection according to Van 't Klooster *et al.*[24] Samples were also injected onto the HPLC systems before flushing through the devices to measure the concentration in the inflow medium (100% values).

Biocompatibility study

The biocompatibility of the plastics and elastomer was tested by culturing HepG2 cells on the substrates. Cells were cultured in the structure shown in Figure 1, and on flat substrates. The substrates were coated with lyophilized rat-tail collagen (Roche, Basel, Switzerland). As control, the cells were also cultivated in tissue culture-treated polystyrene well plates (Corning Costar, Amsterdam, The Netherlands). Cell suspensions containing approximately 120,000 cells were seeded onto the substrates and in the wells. The cells were cultivated for 24 hours in DMEM medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. After 24 hours, the medium was removed and cells were stained with propidium iodide (2 µg/mL) and acridine orange (1 µg/mL) for 4 minutes. Propidium iodide is a membrane impermeable compound which can only enter and stain dead cells by binding to cell-interior compounds, resulting in a red color. Acridine orange can diffuse freely across membranes and stains viable cells green upon exposure to the proper wavelength. After staining, the solution was removed and pictures were taken immediately using a Leica Orthoplan fluorescence microscope (Leica, Wetzlar, Germany) equipped with two filter sets (Ex 465-495, Em 512-558 and Ex 560-590, Em >630). The HepG2 cell experiment was performed in triplicate on three different chips made from the same material.

Results and Discussion

Surface treatment

The treatment of plastic substrates by oxidizing the surface results in a more hydrophilic surface. Due to the treatment, oxygen-containing functional groups are formed onto the surface layer, which results in higher surface free energy and a lower hydrophobicity.[25] This will prevent the adsorption of hydrophobic compounds. However, when the exposure time of ozone is too long, many oxygen containing groups are formed on the surface resulting in a charged surface. This extremely hydrophilic surface will not adsorb hydrophobic compounds, however, electrostatic interaction might occur with the compounds present in the medium. Therefore there is a turn-over point in ozone exposure. The surface should not be exposed too long to ozone, resulting in charged surface, however, too short results in hydrophobic interactions. The surface energy can be measured using contact angle measurements, which give a good indication of the hydrophobicity of a substrate.[18] The preferable contact angle is 40-50°. Substrates with this contact angle will hardly adsorb hydrophobic compounds and will not have electrostatic interactions. The surface of well plates which are tissue-culture treated also has a contact angle of 40-50° (data not shown).

UV ozone

The treatment of the plastics with UV ozone is given in Figure 2. Contact angles were measured 2 hours after treatment for the first set of samples. All substrates show a decrease in contact angle after UV ozone irradiation, indicating increased hydrophilicity. All plastic substrates showed a tremendous decrease in contact angle, and very hydrophilic surfaces were obtained after 60 minutes of treatment. A contact

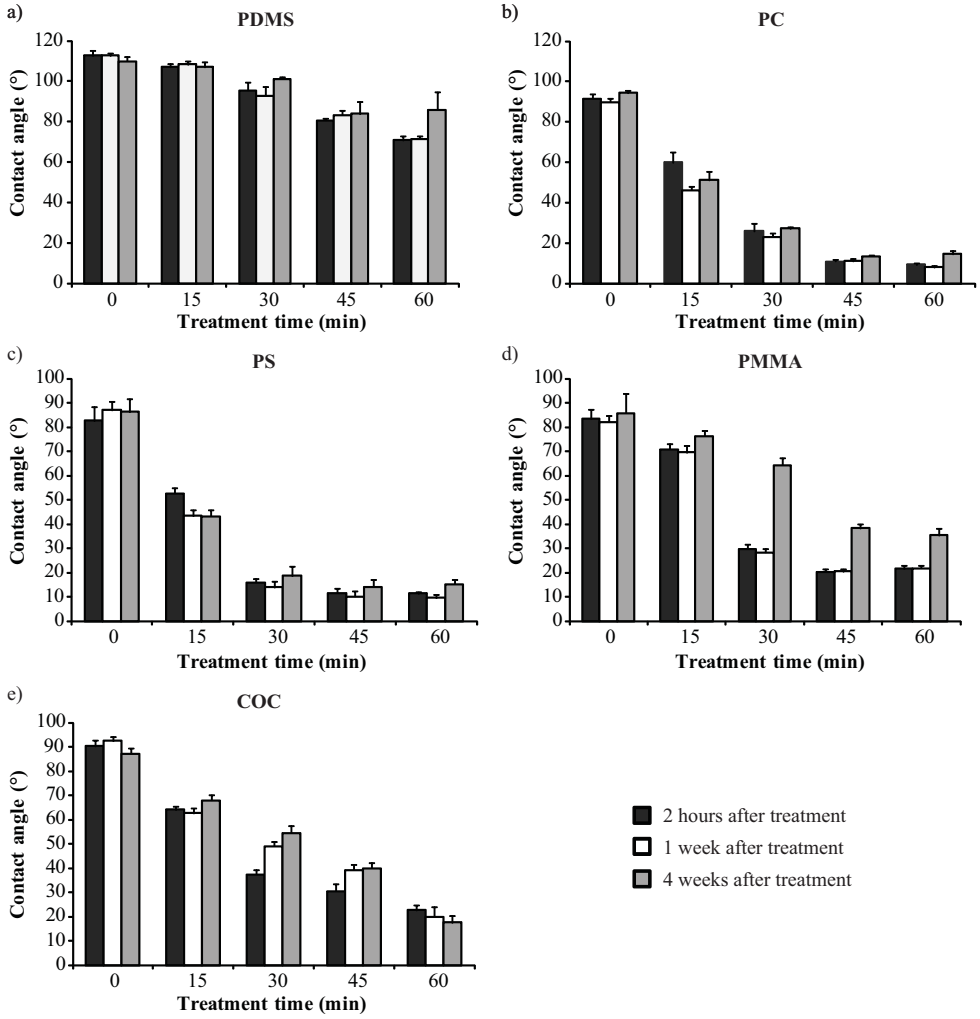

Figure 2. Contact angles of UV ozone-treated (a) PDMS, (b) PC, (c) PS, (d) PMMA, and (e) COC as a function of treatment time. The hydrophobic recovery was measured after one week (white bars) and four weeks (gray bars). Black bars represent the contact angle of the substrates two hours after treatment. Results are average ± standard deviation of 3 separate measurements on three pieces of substrate (nine measurements in total for each type of substrate).

angle of 10° for PC and PS means that many functional oxygen-containing groups are formed, resulting in charged surface which is not beneficial as mentioned above. However, the contact angle of PDMS decreased only from 110° to approximately 70° after 60 minutes of treatment. Probably the contact angle is low directly after treatment as mentioned by others,[14, 15] however, rapid hydrophobic recovery occurs as shown before.[15] It is believed that free siloxanes from the bulk migrate to the surface to recover the surface characteristics to its native form.[19]

PC and PS on the other hand had a contact angle of ~50° already after 15 minutes of treatment. PMMA and COC needed a treatment of 30 minutes to decrease the contact angle to ~50°, as observed before by others.[23] For PC, PS and COC hydrophobic recovery was hardly observed during the 4 weeks measured. However, a relatively high recovery occurred for PMMA 4 weeks after treatment. The surface treated for 30 minutes was almost fully recovered after 4 weeks. Therefore, this substrate can only be used one week after treatment. With the substrates PC and PS hydrophobic recovery was minimal and these substrates can be used for at least four weeks after treatment of 15 minutes with UVOCS. COC does show hydrophobic recovery after treatment for 30 minutes, although after four weeks the contact angle is still ~50°, and therefore COC can also be used for four weeks.

Contact angle measurements after oxygen plasma treatment

Oxygen plasma treatment is harsher compared to UV ozone. Oxygen plasma will produce far more ozone in a given space of time compared to UV ozone, since it contains particles with a high kinetic energy like photons, electrons, ions, radicals, and excited species.[19] Therefore, the exposure time can be much shorter compared to UV ozone treatment to achieve the same contact angles. The exposure time is in seconds compared to minutes with UV ozone. Figure 3 shows the contact angles of the different plastic substrates and PDMS after oxygen plasma treatment. The measurements were performed one hour after treatment for the first set of samples. The contact angles of all substrates decreased also here upon exposure to ozone. PDMS showed a very low contact angle after treatment (<20°). This effect was more pronounced compared to UV ozone, however, this might be due to the fact that contact angles were measured one hour after treatment instead of two hours compared to UV ozone, due to logistic limitations. However, within a week the contact angle of the substrate aged in air almost returned completely to its initial contact angle. This recovery can be reduced when placing the substrates in water after treatment.[26] The hydrophobic recovery was less apparent for the plastics tested. For all four plastic tested, the contact angle decreased to approximately 50° after 30-60 seconds of oxygen exposure. The hydrophobic recovery was comparable to substrates treated with UV ozone, except for polystyrene where the recovery was much higher compared to UV ozone treatment. One week after treatment the contact angle increased by 20°. For

PDMS it was also shown before that hydrophobic recovery was slower when the surface was treated with UV ozone instead of oxygen plasma.[15] According to Berdichevsky *et al.*[15] UV ozone enables much deeper oxidation compared to oxygen plasma. Therefore further studies were performed with UV ozone treatment, where the contact angles were stable for all substrates for at least one week.

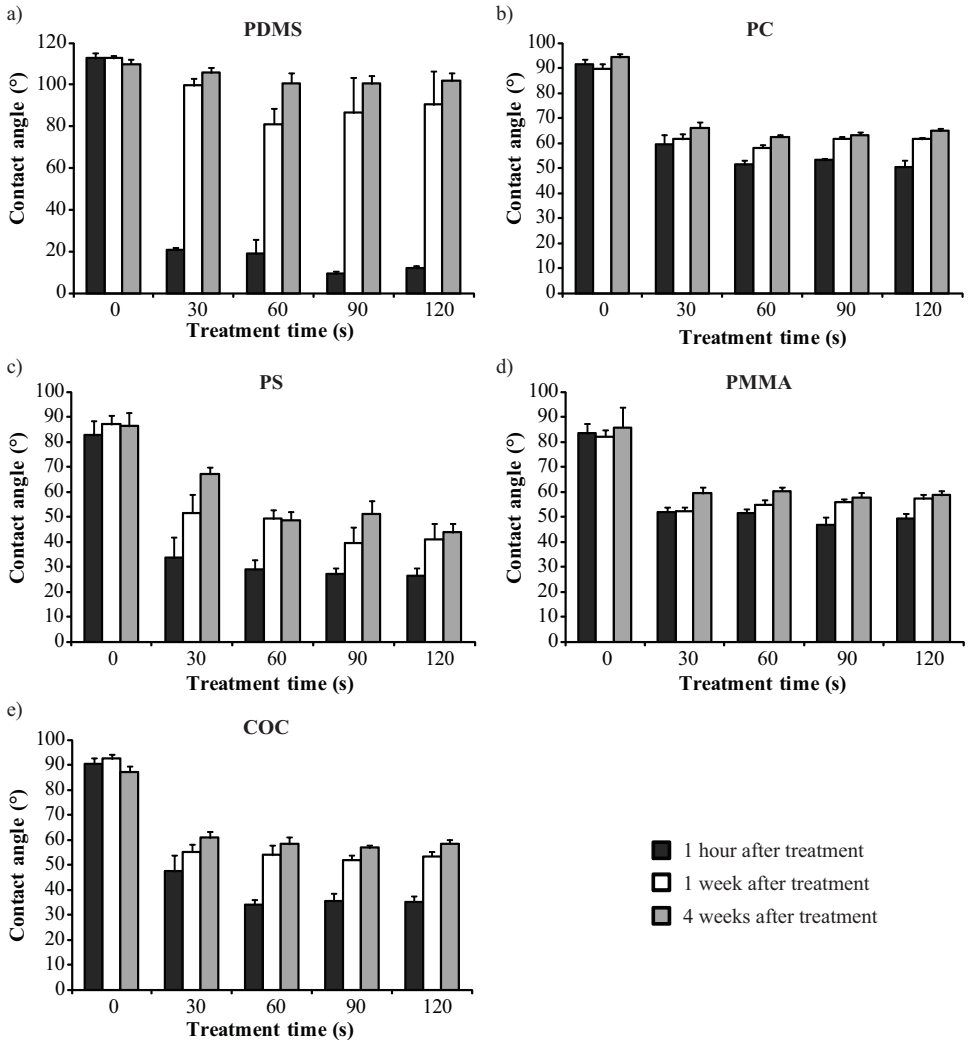

Figure 3. Contact angles of oxygen plasma-treated (a) PDMS, (b) PC, (c) PS, (d) PMMA, and (e) COC as a function of treatment time. The hydrophobic recovery was measured after one week (white bars) and four weeks (gray bars). Black bars represent the contact angle of the substrates one hour after treatment. Results are average ± standard deviation of 3 separate measurements on three pieces of substrate (nine measurements in total for each type of substrate).

Adsorption study

Microfluidic chips were developed using a hot embosser as described above. The structure, given in Figure 1, consisted of an inlet channel, outlet channel, and a chamber for the hepatocyte culture. The structure was patterned in plastic using a brass mold. Thereafter the pieces of plastics with structure and flat pieces to seal the device were placed in the UV ozone to oxidize the surfaces. PS and PC were treated for 15 minutes, and COC and PMMA were treated for 30 minutes. Thereafter the plastic pieces were again placed in the hot embosser for thermal bonding. Due to the UV ozone treatment, the glass transition temperature (T_g) of the plastics decreased at the surface layer, while the bulk polymer retained the T_g.[25, 27, 28] This resulted in thermal bonding at temperatures below the glass transition point (see Table 1) without deformation of the structure.

The chips were placed under a microscope and perfused with medium with a flow rate up to 10 µL/min, and no leakage was observed for at least 24 hours (data not shown). Thermally bonded chips were placed in a humidified incubator set at 37°C, and flushed with syringes filled with medium containing 7-ethoxycoumarin (7-EC), 7-hydroxycoumarin (7-HC), 7-hydroxycoumarin glucuronide (7-HC-G) and 7-hydroxycoumarin sulfate (7-HC-S) at a flow rate of 5 µL/min. Medium was collected during two hours of perfusion and the concentration of 7-EC and its metabolites was measured in the 600 µL collected fraction. Recoveries of the compounds were calculated from the initial concentrations of 7-EC and its metabolites present in the

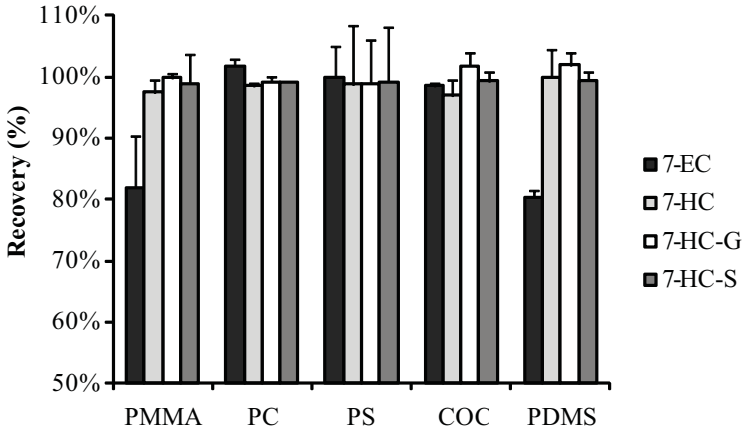

Figure 4. Recovery of 7-ethoxycoumarin (7-EC) and its metabolites 7-hydroxycoumarin (7-HC), 7-hydroxycoumarin glucuronide (7-HC-G), and 7-hydroxycoumarin sulfate (7-EC-S) measured at the outlet as a percentage of the initial concentration after 2 hours flushing through the device at 5 µL/min. Substrates were pretreated with UV ozone (15 minutes for PS, PC and PDMS; 30 minutes for PMMA and COC). Results are average ± standard deviation of 3 separate devices for each substrate.

medium before perfusion. The recoveries of the metabolites and substrate during 2 hours of perfusion are given in Figure 4. For all plastics and PDMS no adsorption of the relatively hydrophilic 7-hydroxycoumarin and the charged metabolites 7-hydroxycoumarin glucuronide and 7-hydroxycoumarin sulfate was observed. However, the recovery of 7-ethoxycoumarin was lower in PMMA and PDMS after perfusion. Only 80% of the initial value was found at the outlet of the device. This indicated adsorption of 7-ethoxycoumarin onto the wall of the PMMA and PDMS structures, as observed before for PDMS.[6]

The chips were also flushed with medium containing testosterone with its metabolites for two hours, similar as for 7-ethoxycoumarin. Recoveries were calculated from the 600 µL collected medium and the concentration of testosterone and it metabolites present in the medium before perfusion (Figure 5). Again the substrates PC, PS, and COC did not adsorb the compound and its metabolites. One hundred percent recovery was obtained for all compounds. In case of PMMA, the amount of androstenedione and the hydroxytestosterone metabolites at the outlet of the chip were determined at a concentration similar as the initial value, however, testosterone had a lower recovery. Only 85% of testosterone was found back at the outlet of the chip. Again adsorption of hydrophobic compounds at the channel wall of PMMA was found, as described for 7-ethoxycoumarin. PDMS showed also adsorption of testosterone and its metabolites. Only 6β-hydroxytestosterone, the most hydrophilic metabolite, had a recovery of 100%. 16α and 2α had a recovery of only

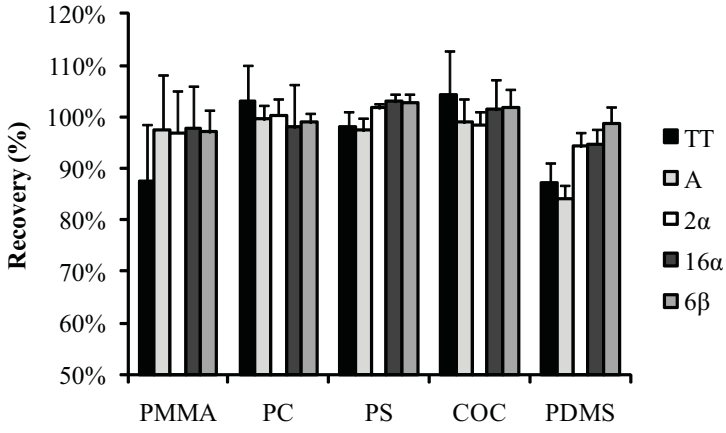

Figure 5. Recovery of testosterone (TT) and its metabolites androstenedione (A), 2α-hydroxytestoterone, 16α-hydroxytestosterone, and 6β-hydroxytestosterone measured at the outlet as a percentage of the initial concentration after 2 hours flushing through the device at 5 µL/min. Substrates were pretreated with UV ozone (15 minutes for PS, PC and PDMS; 30 minutes for PMMA and COC). Results are average ± standard deviation of 3 separate devices for each substrate.

95%, and around 15% of testosterone and androstenedione were adsorbed onto the PDMS, or maybe absorbed into PDMS.[8] These results indicate that only PC, PS, and COC from the tested substrates are resistant to adsorption of hydrophobic compounds after UV ozone treatment.

Biocompatibility study

Toxicity can be caused by the peroxides formed by UV ozone treatment, which remain present for several days.[29] Previously it was shown that PMMA[30], PS[31], PC[32], COC[33], and PDMS[34] are biocompatible for HepG2 cells. However, most of these studies were performed without surface treatment, and few experiments have been reported in which different substrates were compared with each other.

PC and PS substrates were treated for 15 minutes with UV ozone, and PMMA and COC for 30 minutes, similar as for the adsorption experiment. HepG2-cells were cultured on substrates without collagen coating, and adhered to the plastic. Many cells were adhered to COC, PC and PS, and also grow on the surface (data not shown). On PMMA less cells were present after 24 h, and ca 25% of the cells appeared non viable (results not shown). This may indicate that either the cells do not adhere or they do not proliferate on PMMA and/or they were released from the surface due to the loss of viability. On PDMS hardly any cells were attached, although the few cells that did adhere to the surface were all viable.

Normally, cell culture plates are coated with collagen to improve the initial adhesion of HepG2 cells on the substrates. Collagen is a natural extracellular matrix component with low immunogenicity, promoting cell adhesion and proliferation.[35] Figure 6 shows the HepG2 cells cultured on the different plastics, on PDMS and in well plates after collagen coating. As can be seen in the figures, many intact cells are attached to the surface. With collagen coating also many cells attached to the hydrophobic PDMS surface, and hardly any dead cells were visible. Again very similar results were obtained for cells cultured on PC, PS, COC and in well plates (control), with only a few dead cells (~2%). On the other hand, many cells adhered to PMMA after collagen coating, however, again many dead cells were visible. Around 11% of the cells were not viable (see Figure 6g). Therefore the treatment of PMMA with UV ozone does not result in a biocompatible surface to cultivate liver cells. The other plastics were all biocompatible after treatment.

Conclusion

Culturing cells in microfluidic devices is a powerful tool to be able to study cellular functions under conditions that mimic the *in vivo* situation. Many substrates can be used to fabricate microfluidic devices; however, not all of them are biocompatible. The main question of this study was to find out which plastics were suitable for the cultivation of cells or tissue in a microfluidic device with low toxicity and low

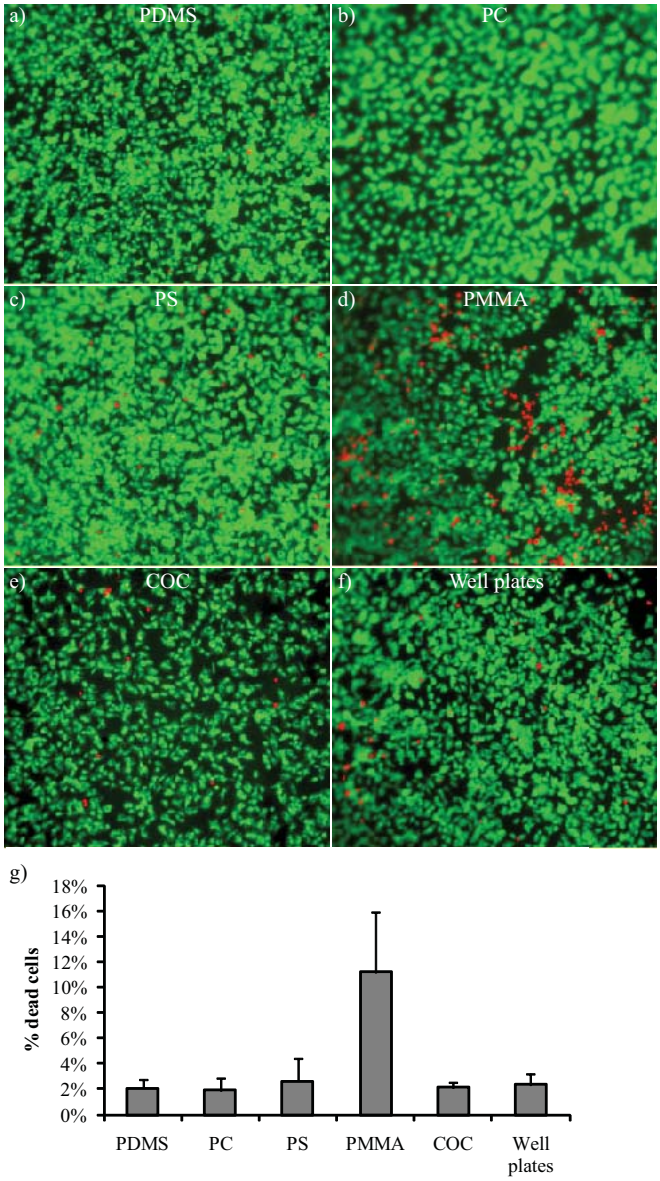

Figure 6. Viability of HepG2 cells cultured on polymers after UV ozone. Life cells are stained green (by acridine orange) and dead cells are red (by propidium iodide). The polymers tested are (a) PDMS, (b) PC, (c) PS, (d) PMMA, (e) COC, and (f) well plates (polystyrene). (g) The amount of dead cells as percentage of the total amount of cells present. Results are average ± standard deviation of 3 separate devices for each substrate measured for three different HepG2 passages (nine measurements in total for each type of substrate). PC and PS were treated with UV ozone for 15 minutes, and PMMA, COC and PDMS were treated for 30 minutes.

adsorption of substrates and metabolites. Here the use of polydimethylsiloxane (PDMS), polymethylmethacrylate (PMMA), polystyrene (PS), polycarbonate (PC), and cyclic olefin copolymer (COC) was tested for suitability in cell and tissue applications. The native surface of these materials is relatively hydrophobic; however, surfaces can be easily altered through oxidation to become more hydrophilic. It was shown that both UV ozone and oxygen plasma can be used to increase the hydrophilicity of the materials tested. An additional advantage of oxidizing the plastic surfaces appeared the lowered glass transition temperature of the surface, which facilitated the bonding of the substrates at temperatures below the glass transition point of the bulk of the device, preventing deformation of the microfluidic structure.

UV ozone treatment is preferred over oxygen plasma treatment because the hydrophilic surface was more stable after treatment compared to oxygen plasma. After UV ozone treatment of the materials which creates a contact angle of ~50°, hydrophobic substrates were hardly adsorbed onto the materials. Solutions containing 7-ethoxycoumarin or testosterone and their metabolites were flushed through devices made of PS, PC and COC without measurable absorption, whereas PDMS and PMMA exhibited a lower recovery of the hydrophobic compounds.

After collagen coating, HepG2 cells adhered to the surface of all materials with high viability, except for PMMA. The UV ozone treatment of PDMS, PC, PS and COC did not result in toxicity, and showed a dense network of viable cells on the surface 24 hours after seeding. However, on surfaces made of UV ozone treated and untreated PMMA a considerable amount of dead cells were visible.

The chemical resistance of PC and COC is far better than PS.[28] When devices are flushed with organic solvents, which are used for on-line introduction of liquid chromatography on chip for example, the use of PC or COC is recommended. COC has the additional advantage compared to PC of exhibiting a lower autofluorescence, which is beneficial for optical imaging.[36] In conclusion, microfluidic devices made from PC after oxidizing the surface for 15 minutes, or COC after 30 minutes of oxidation, are suitable for the incorporation of cells or tissue and will allow the low-cost production of a biocompatible device with low-adsorption profile for both hydrophobic and hydrophilic compounds. Structures can be easily patterned by using a hot embosser or by injection molding.[37]

Acknowledgements

The authors would like to thank Hella Logtenberg and Dr. Wesley R. Browne (Stratingh Institute for Chemistry, University of Groningen) for the use of their apparatus to measure contact angles. We also would like to thank Marieke van Beuge and Catharina Reker-Smit for their help with the HepG2 cells.

References

1. Dittrich, P. S.; Manz, A., Lab-on-a-chip: Microfluidics in drug discovery. *Nat. Rev. Drug Discov.* 2006, **5** (3), 210-218.
2. Meyvantsson, I.; Beebe, D. J., Cell culture models in microfluidic systems. *Annu. Rev. Anal. Chem.* 2008, **1** (1), 423-449.
3. El-Ali, J.; Sorger, P. K.; Jensen, K. F., Cells on chips. *Nature* 2006, **442** (7101), 403-411.
4. Lee, P. J.; Hung, P. J.; Lee, L. P., An artificial liver sinusoid with a microfluidic endothelial-like barrier for primary hepatocyte culture. *Biotechnol. Bioeng.* 2007, **97** (5), 1340-1346.
5. Zhou, J. W.; Ellis, A. V.; Voelcker, N. H., Recent developments in PDMS surface modification for microfluidic devices. *Electrophoresis* 2010, **31** (1), 2-16.
6. Van Midwoud, P. M.; Groothuis, G. M. M.; Merema, M. T.; Verpoorte, E., Microfluidic biochip for the perifusion of precision-cut rat liver slices for metabolism and toxicology studies. *Biotechnol. Bioeng.* 2010, **105** (1), 184-194.
7. Duffy, D. C.; McDonald, J. C.; Schueller, O. J. A.; Whitesides, G. M., Rapid prototyping of microfluidic systems in poly(dimethylsiloxane). *Anal. Chem.* 1998, **70** (23), 4974-4984.
8. Toepke, M. W.; Beebe, D. J., PDMS absorption of small molecules and consequences in microfluidic applications. *Lab Chip* 2006, **6** (12), 1484-1486.
9. Prieto, A.; Basauri, O.; Rodil, R.; Usobiaga, A.; Fernandez, L. A.; Etxebarria, N.; Zuloaga, O., Stir-bar sorptive extraction: A view on method optimisation, novel applications, limitations and potential solutions. *J. Chromatogr. A* 2010, **1217** (16), 2642-2666.
10. Lancas, F. M.; Queiroz, M. E. C.; Grossi, P.; Olivares, I. R. B., Recent developments and applications of stir bar sorptive extraction. *J. Sep. Sci.* 2009, **32** (5-6), 813-824.
11. Wu, D. P.; Luo, Y.; Zhou, X. M.; Dai, Z. P.; Lin, B. C., Multilayer poly(vinyl alcohol)-adsorbed coating on poly(dimethylsiloxane) microfluidic chips for biopolymer separation. *Electrophoresis* 2005, **26** (1), 211-218.
12. Witek, M. A.; Wei, S. Y.; Vaidya, B.; Adams, A. A.; Zhu, L.; Stryjewski, W.; McCarley, R. L.; Soper, S. A., Cell transport via electromigration in polymer-based microfluidic devices. *Lab Chip* 2004, **4** (5), 464-472.
13. Amstein, C. F.; Hartman, P. A., Adaptation of plastic surfaces for tissue culture by glow discharge. *J. Clin. Microbiol.* 1975, **2** (1), 46-54.
14. Kim, J.; Chaudhury, M. K.; Owen, M. J., Hydrophobic recovery of polydimethylsiloxane elastomer exposed to partial electrical discharge. *J. Colloid Interface Sci.* 2000, **226** (2), 231-236.
15. Berdichevsky, Y.; Khandurina, J.; Guttman, A.; Lo, Y. H., UV/ozone modification of poly(dimethylsiloxane) microfluidic channels. *Sens. Actuator B-Chem.* 2004, **97** (2-3), 402-408.
16. Schift, H.; David, C.; Gabriel, M.; Gobrecht, J.; Heyderman, L. J.; Kaiser, W.; Koppel, S.; Scandella, L., Nanoreplication in polymers using hot embossing and injection molding. *Microelectron. Eng.* 2000, **53** (1-4), 171-174.
17. De Mello, A., Plastic fantastic? *Lab Chip* 2002, **2** (2), 31N-36N.
18. Churaev, N. V., Contact angles and surface forces. *Adv. Colloid Interface Sci.* 1995, **58** (2-3), 87-118.
19. Olah, A.; Hillborg, H.; Vancso, G. J., Hydrophobic recovery of UV/ozone treated poly(dimethylsiloxane): Adhesion studies by contact mechanics and mechanism of surface modification. *Appl. Surf. Sci.* 2005, **239** (3-4), 410-423.
20. Morra, M.; Occhiello, E.; Garbassi, F., Hydrophobic recovery and misting behavior of plasma treated ps and pc surfaces. *Angew. Makromol. Chem.* 1991, **189** (1), 125-136.
21. Occhiello, E.; Morra, M.; Cinquina, P.; Garbassi, F., Hydrophobic recovery of oxygen-plasma-treated polystyrene. *Polymer* 1992, **33** (14), 3007-3015.
22. Constantinides, G.; Tweedie, C. A.; Holbrook, D. M.; Barragan, P.; Smith, J. F.; Van Vlietl, K. J., Quantifying deformation and energy dissipation of polymeric surfaces under localized impact. *Mater. Sci. Eng. A-Struct. Mater. Prop. Microstruct. Process.* 2008, **489** (1-2), 403-412.
23. Tsao, C. W.; Hromada, L.; Liu, J.; Kumar, P.; DeVoe, D. L., Low temperature bonding of PMMA and COC microfluidic substrates using UV/ozone surface treatment. *Lab Chip* 2007, **7** (4), 499-505.
24. Van 't Klooster, G. A. E.; Blaauboer, B. J.; Noordhoek, J.; Van Miert, A., Cytochrome P450 induction and metabolism of alkoxyresorufins, ethylmorphine and testosterone in cultured hepatocytes from goats, sheep and cattle. *Biochem. Pharmacol.* 1993, **46** (10), 1781-1790.
25. Bhattacharyya, A.; Klapperich, C. M., Mechanical and chemical analysis of plasma and ultraviolet-

ozone surface treatments for thermal bonding of polymeric microfluidic devices. *Lab Chip* 2007, **7** (7), 876-882.
26. Bausch, G. G.; Stasser, J. L.; Tonge, J. S.; Owen, M. J., Behavior of plasma-treated elastomeric polydimethylsiloxane coatings in aqueous environment. *Plasma Polym.* 1998, **3** (1), 23-34.
27. Truckenmuller, R.; Henzi, P.; Herrmann, D.; Saile, V.; Schomburg, W. K., Bonding of polymer microstructures by UV irradiation and subsequent welding at low temperatures. *Microsyst. Technol.* 2004, **10** (5), 372-374.
28. Tsao, C. W.; DeVoe, D. L., Bonding of thermoplastic polymer microfluidics. *Microfluid. Nanofluid.* 2009, **6** (1), 1-16.
29. Welle, A.; Gottwald, E., UV-based patterning of polymeric substrates for cell culture applications. *Biomed. Microdevices* 2002, **4** (1), 33-41.
30. Sakai, Y.; Nakazawa, K., Technique for the control of spheroid diameter using microfabricated chips. *Acta Biomater.* 2007, **3** (6), 1033-1040.
31. Bokhari, M.; Carnachan, R. J.; Cameron, N. R.; Przyborski, S. A., Culture of HepG2 liver cells on three dimensional polystyrene scaffolds enhances cell structure and function during toxicological challenge. *J. Anat.* 2007, **211** (4), 567-576.
32. Altmann, B.; Giselbrecht, S.; Weibezahn, K. F.; Welle, A.; Gottwald, E., The three-dimensional cultivation of the carcinoma cell line HepG2 in a perfused chip system leads to a more differentiated phenotype of the cells compared to monolayer culture. *Biomed. Mater.* 2008, **3** (3), 10.
33. Mehta, G.; Lee, J.; Cha, W.; Tung, Y. C.; Linderman, J. J.; Takayama, S., Hard top soft bottom microfluidic devices for cell culture and chemical analysis. *Anal. Chem.* 2009, **81** (10), 3714-3722.
34. Leclerc, E.; Sakai, Y.; Fujii, T., Cell culture in 3-dimensional microfluidic structure of PDMS (polydimethylsiloxane). *Biomed. Microdevices* 2003, **5** (2), 109-114.
35. Friess, W., Collagen - biomaterial for drug delivery. *Eur. J. Pharm. Biopharm.* 1998, **45** (2), 113-136.
36. Piruska, A.; Nikcevic, I.; Lee, S. H.; Ahn, C.; Heineman, W. R.; Limbach, P. A.; Seliskar, C. J., The autofluorescence of plastic materials and chips measured under laser irradiation. *Lab Chip* 2005, **5** (12), 1348-1354.
37. Fiorini, G. S.; Chiu, D. T., Disposable microfluidic devices: Fabrication, function, and application. *Biotechniques* 2005, **38** (3), 429-446.